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(54) Title: PRODUCTS AND METHODS FOR THE DETECTION OF PERIODONTAL DISEASE

(57) Abstract

A method for diagnosing periodontal disease is disclosed. The method involves providing a substrate coated with a plurality of discrete spots, each spot containing one type of an immobilized periodontopathogenic bacterium in a predetermined concentration; contacting the immobilized bacterium with a sample containing blood from a patient suspected of having periodontal disease, thereby forming conjugates of antibodies with the immobilized bacterium; and detecting the antibody conjugates; wherein the spots containing immobilized bacterium comprise at least one spot containing *Actinobacillus actinomycetemcomitans, serotype B* or *Porphyromonas gingivalis, serotype A*. Substrates and kits are also provided.

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PRODUCTS AND METHODS FOR THE DETECTION OF PERIODONTAL DISEASE

This application is a continuation-in-part of U.S. Serial No. 07/866,128 filed April 7, 1992, which is a continuation of U.S. Serial No. 07/320,997 filed March 9, 1989.

Field of the Invention

This invention relates to the detection of periodontal disease, particularly to improvements in rapid detection and assessment of periodontal disease by determining the levels of antibodies to specific periodontal pathogens.

Background of the Invention

Microbiological studies of periodontal disease in humans have supported the concept of a specific bacterial etiology. Numerous Gram-negative members of the subgingival microflora have been implicated as causative agents. In addition, the existence of a host immune response to oral microorganisms and its relationship to periodontal disease has been examined in numerous studies. In general, these reports have suggested that elevations in the cell-mediated or humoral response to certain plaque-associated microorganisms is noted with increasing severity of periodontal disease. Evidence has indicated that different forms of periodontal disease have a distinctive microflora colonizing the disease sites, for example, **A. actinomycetemcomitans** in localized juvenile periodontitis and **P. gingivalis** in adult and generalized forms of periodontitis. See, for example, Ebersole et al., **Clin. Exp. Immunol.** 47:43-52 (1981); Ebersole et al., **J. Clin. Immunol.** 3:321-331 (1983); Ebersole et al., **J. Perio. Res.** 17:478-480 (1982); Listgarten et al., **J. Clin. Periodontal.** 8:155-164 (1981); Ebersole et al., **Infec. Immun.** 51:507-517 (1986); Mouton et al., **Infect. Immun.** 31:182-192 (1981).

Although studies from a number of laboratories have consistently implicated these microorganisms in disease, additional evidence has indicated that disease in selected individuals may be associated with other bacteria, including **P. intermedia**, **E. corrodens**, **F. nucleatum**, **C. rectus (formerly W. recta)**, **Eubacterium spp.**, **Capnocytophaga spp.**, **T. denticola**, **B. forsythus**, and **Selenomonas spp.** See for example, Naito et al., **Infect. Immun.** 45:47-51 (1984); Genco et al., **Anaerobic Bacteria; Selected Topics,** Plenum Press, New York, pp. 277-293 (1980); Williams et al., **Infect. Immun.** 49:742-750 (1985); Cheng et al., **J. Perio. Res.** 20:602-612 (1985); Sandholm et al., **J. Clin. Periodontal.** 13:646-650 (1986); Lai et al., **J. Clin. Periodontal.** 13:752-757 (1986); Naito et al., **J. Dent. Res.** 64:1306-1310 (1985); Vincent et al., **J. Periodontal** 57:625-631 (1986); Tew et al., **J. Perio. Res.** 20:580-590 (1985); Tolo et al., **J. Perio. Res.** 20:113-121 (1985); Vincent et al., **J. Periodontal.** 56:464-469 (1985); Tew et al., **Infect. Immun.** 48:303-311 (1985).

Cross-sectional studies of human immune responses in periodontal disease have been used to delineate distinctive systemic antibody response patterns in diseased patients. Abnormal antibody patterns have been used to direct microbiological studies investigating the presence of the specific bacteria in disease active and inactive sites. The results showed that using the systematic antibody level as a stratifying principal predicts a colonization of the microorganisms in the subgingival plaque. Other studies have extended these findings to correlate the antibody response patterns with the clinical parameters of disease and have shown particular symptoms associated with those patterns. These latter studies have described the association of the antibody patterns with the progression of disease and response to treatment, and in longitudinal

studies have demonstrated fluctuations in systemic antibody levels that correlate with treatment success and apparent reactivation of disease. See Haffajee et al., **J. Clin. Periodontal.** 12: 533:-567 (1985); Ebersole et al., **Oral Micro. Immunol.** 2:53-59 (1987); Ebersole et al., **J. Perio. Res.** 22-184-186 (1987).

In copending application Serial No. 07/866,128, filed April 7, 1992, a rapid test for determining elevated antibody to periodontitis-associated bacteria was described. The technique utilizes dot-immunoblotting, i.e. dot-blot (DB) assay, on nitrocellulose paper with whole cell antigen preparations from formalinized specific periodontopathogenic bacteria chosen from a group consisting of **Actinobacillus actinomycetemcomitans, Porphyromonas (Bacteriodes) gingivalis, Prevotella intermedia (Bacteriodes intermedius), Bacteriodes forsythus, Capnocytophaga spp., Eikenella corrodens, Eubacterium spp., Fusobacterium nucleatum, Selenomonas spp.**, and **Campylobacter rectus (Wolinella recta)**.

Improvements providing rapid, sensitive procedures for diagnosing and assessing periodontal disease by detecting abnormal levels of antibody to specific bacteria in a patient's serum are desired.

Summary of the Invention

The present invention provides improved methods, compositions and kits for rapid, sensitive procedures for diagnosing and assessing periodontal disease. In accord with the invention, serum antibodies to specific serotypes of particular periodontopathogenic bacteria are detected. Thus, one embodiment of the present invention provides a method for diagnosing periodontal disease, the method comprising: providing a substrate coated with a plurality of discrete spots, each spot containing an immobilized periodontopathogenic bacterium in a predetermined

concentration; contacting the immobilized bacterum with a sample of blood to conjugate antibodies to the bacterium; and detecting the conjugated antibodies, wherein the spots containing immobilized bacterium include at least one spot containing a bacterium selected from **Actinobacillus actinomycetemcomitans, serotype B** and **Porphyromonas gingivalis, serotype A**. The use of these serotypes have been found to increase the accuracy of diagnosis using the assay.

In another embodiment of the invention, a solid substrate is provided having coated thereon a plurality of discrete spots in a predetermined arrangement, each spot containing an immobilized periodontopathogenic bacterium in a predetermined concentration, wherein the spots include a spot containing a bacterium selected from **Actinobacillus actinomycetemcomitans, serotype B** and **Porphyromonas gingivalis, serotype A**.

In another embodiment of the invention, after contacting a selected spot having immobilized bacterium with a blood sample, the antibodies from the sample are fixed to the bacterial antigens, for example, with methanol. Such fixation has been found to increase the sensitivity of the assay and can also increase the accuracy.

In a preferred embodiment of the invention, the plurality of discrete spots of immobilized bacteria include a spot containing one of each of the following bacterium: **Actinobacillus actinomycetemcomitans, serotype B** and **Porphyromonas gingivalis, serotype A, Campylobacter rectus, Prevotella intermedia** and **Eikenella corrodens.**

The invention also provides a substrate and kit for use in performing dot-blot immunoassay. A solid substrate is provided containing a predetermined arrangement of discrete spots, each input containing one type of an immobilized periodontopathogenic bacterium in a predetermined

concentration, wherein the spots containing immobilized bacterium comprise at least one spot containing **A. actinomycetemcomitans, serotype B** or **P. gingivalis, serotype A**. Kits in accord with the invention include the substrate, preferably a nitrocellulose sheet, and one or more of the following: a reference sample-containing a predetermined quantity of antibodies to specific bacterium, labelled anti-human IgG, a chromophase substrate for an enzyme label, and a solution of methanol in buffered saline.

In accord with the invention, periodontal disease can be assessed by determining the etiology of the disease using the methods, compositions and kits of the invention. This enables treatment to be specific to the etiology of the particular patient and to be changed as treatment progresses. It further permits the treatment progress or success to be evaluated in a semi-quantitative manner.

Detailed Description of the Invention

In accord with the present invention periodontopathogens are immobilized on a substrate. The particular bacteria used are selected from those pathogens to which have been found elevated levels of systemic antibodies in patients having periodontal disease such as, for example, **Actinobacillus actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg), Prevotella intermedia (Pi), Bacteriodes forsythus, Capnocytophaga spp., Eikenella corrodens (Ec), Eubacterium spp., Fusobacterium nucleatum (Fn), Selenomonas spp.**, and **Campylobacter rectus (Cr)**. In accord with the present invention, the bacteria used include at least one of **Actinobacillus actinomycetemcomitans, serotype B (Aa, B)** and **Porphyromonas gingivalis, serotype A, (Pg, A)** preferably both.

Specifically, the **A. actinomycetemcomitans, serotype B** is defined by strain Y4 originally designated at Forsyth Dental Center and currently available from the American Type

Culture Collection (ATCC) as ATCC 43718 submitted by H. Reynolds from S.S. Socransky and the **P. gingivalis, serotype A** is defined by strain ATCC 33277 which is the Type strain for this species submitted to the American Type Culture Collection by A.L. Coykendall as strain 2561 from J. Slots. These serotypes have been defined by using polyclonal rabbit antibody and murine monoclonal antibodies which have the capability to separate **A. actinomycetemcomitans** and **P. gingivalis** strains into three serotypes for each species. See Zambon, J.J. et al., **J. Periodontal**, 54:707-711 (1983), Asikainen, S. et al., **Oral Micro. Immunol.** 6:115-118 (1991), Zambon, J.J. et al., **Infect. Immun.** 44:19-27 (1983), Ebersole, J.L. et al., **Oral Micro. Immunol.** 2:53-59 (1987); Gmur, R., et al., **Oral Micro. Immunol.** 3:181-186, (1988), Hanazawa, S. et al., **Infect. Immun.** 46:285-287 (1984), Naito, Y. et al., **Infect. Immun.** 50:231-235 (1985), Nagata, A. et al., **J. Perio. Res.** 26:184-190 (1991), Ebersole, J.L. et al., **J. Clin. Micro.** 19:639-644 (1984).

The immobilized bacteria are contacted with a sample of blood from a patient suspected of having periodontal disease. Antibodies to the periodontopathogens that are present in the blood complex with the immobilized bacterium. The complexes can be detected using labelled antisera by conventional techniques well known to those skilled in the art such as, for example, ELISA and DB techniques.

Preferably, the periodontopathogens are immobilized on a solid substrate by placing discrete spots of a selected bacteria on a substrate such as, for example, nitrocellulose. Other conventional substrates can also be used.

The concentration of bacteria in each spot is chosen such that after being incubated with the patients's blood, detection of bound antibody will produce a positive result if abnormal levels of the particular antibody are present in

the patient's blood, the particular concentrations of bacterial antigen in each spot will depend not only on the levels of serum antibodies found to correspond to certain clinicopathological states of periodontal disease, but also on the sensitivity of the method used to assay for the bound antibody. A concentration of 10^5 to 10^9 organisms per 10 microliters can be utilized to detect serum antibodies using a nitrocellulose substrate.

To detect the levels of antibodies in the blood of a patient, a sample of the blood is drawn, preferably either venous or capillary blood. Most preferably, capillary blood is used. The blood is typically diluted, about 1:10 to 1:200 has been found convenient. However, the dilution ratio should be adjusted based on the level of antibody to be detected and the sensitivity of the detection system. The diluent can be any suitable solution that does not interfere with the assay. Typically, saline or a physiological buffer is used. Surfactant and an anticoagulant or other additives may also be used as long as they do not interfere with the assay. A convenient diluent is made of phosphate buffered saline (PBS) having about 0.01 to 0.5% Tween-20 (trademark) surfactant, preferably with sodium azide in an amount sufficient to prevent contamination.

The bacterial antigen/antibody conjugate can be detected by any method known to those skilled in the art. Conveniently, labelled anti-human sera, for example, labelled goat anti-human IgG is used. The label can be any conventional label capable of detection by methods well known to those skilled in the art. For example, detectable components that can be used in the present invention, included dyes, pigments and complexes detectable by fluorescence measurement; emission tags; radioactive tags; chemical reagents; antigens; haptens, immunological species

such as antibodies and antibody-antigen conjugates; enzymes; and precursors and reaction products of said components. The use of such components is well known to the person of ordinary skill in the art.

Preferably, an enzyme is attached to the anti-sera and the enzyme is detected by reaction that produces a visible or color change, the intensity of which is proportional to the level of antibody in the sample. Typical examples of enzymes and their substrates/chromophores useful in the present invention include:

Alkaline phosphatase - - - - BCIP/NBT

Horseradish peroxidase - - - 4-chloro-1-naphthol & H_2O_2

b-Galactosidase - - -BCI-beta-d-galactopyranoside (X-Gal)

Glucose Oxidase - - -Glucose-p-NBT-PMS

In a preferred embodiment of the invention, nitrocellulose sheet is used as a solid substrate for the pathogens. The nitrocellulose sheet is hydrated in PBS. Ten microliter aliquots of formalized bacteria, whole cell antigen preparation, are dispensed in a row and the sheet incubated for 5 to 30 minutes, preferably 10 to 15 minutes. The substrate is washed with PBS and allowed to dry. Non-specific binding sites are blocked by incubating with a suitable blocking agent, e.g., powdered milk in PBS (5%), bovine serum albumin (BSA), etc., for about 30 minutes to about 2 hours, after which the sheet is again rinsed and allowed to dry.

Preferably, a positive and a negative control are included on the sheet.

To conduct the assay, the sheet having immobilized bacterial antigen is hydrated. Aliquots of the diluted patient blood sample are applied to each spot of immobilized antigen and the sheet is incubated for about 5 minutes to about 1 hour and then rinsed. The nitrocellulose sheet is

then washed and fixed in methanol for about 5 to about 30 minutes.

After washing, the sheet is saturated with a goat anti-human IgG HRP (horseradish peroxidase conjugate) and incubated for about 5 minutes to about 2 hours. After washing, the amount of enzyme remaining, i.e. fixed to the bacterial antigen, is detected using a chromophore substrate such as 4-chloro-1-naphthol and hydrogen peroxide for about 1 to about 5 minutes. A visible color develops having an intensity proportional to the level of antibody against the specific pathogen in the patient's blood sample. In this manner, a threshold quantity of IgG in the patient's blood having a specific binding affinity for the particular bacterial antigen is detected.

Whether the threshold quantity of IgG in the patient's blood is exceeded is determined by comparing the color change produced by the patient's blood with that produced by a positive reference serum. For each periodontal pathogen, a positive reference serum is made by obtaining a pool of serum from patients with periodontal disease. The antibody level in each reference serum is determined by an ELISA to be two standard deviations above the antibody level in a normal population. For each serum sample, an ELISA is performed using each periodontopathogen according to the method described in Ebersole et al., **J. Perio. Res.** 15:621-632 (1980), which is hereby incorporated by reference.

Each reference serum sample is diluted according to the particular periodontopathogen, for example:

A. actinomycetemcomitans	1:400
P. intermedia	1:400
E. corrodens	1:400
Capnocytophaga spp.	1:200
F. nucleatum	1:200
P. gingivalis	1:100

C. rectus	1:100
Eubacterium spp.	1:100
Selenomonas spp.	1:100

The ELISA is performed for antibodies to each particular pathogen, for example, on dilutions of the serum samples as set forth above. If the ELISA for each sample exhibits an optical density at 410 nm of 1.2-1.4 using alkaline phosphatase developing reagents and p-nitrophenylphosphate as substrate, that sample is deemed a suitable reference serum for the particular periodontopathogen. That is, it represents an antibody level to the particular periodontopathogen which is approximately two standard deviations above the mean level in the general population. The DB colorimetric assay is then performed with the reference serum in the same manner as the patient's serum to produce a reference spot.

The following examples are described to further illustrate the invention. In the examples, all steps are carried out at room temperature unless otherwise noted.

Preparative Example 1

Nitrocellulose sheets are coated with discrete spots of each of the following periodontopathogenic bacteria: **Actinobacillus actinomycetemcomitans**, **Porphyromonas gingivalis**, **Prevotella intermedia**, **Eikenella corrodens**, and **Campylobacter rectus**. The bacteria are killed and preserved in formalin and then immobilized on the nitrocellulose matrix by placing a 5μl drop containing 10^7 to 10^8 organisms of each specific bacteria on the nitrocellulose sheet in rows containing a spot for each bacteria.

A sample of the patient's blood is extracted which may be either venous or capillary blood. The blood is diluted 1:50 in PBS having 0.05% Tween 20 and NaN_3. 10μl aliquots of the diluted blood sample are then applied to the discrete bacterial spots and incubated for four minutes at room

temperature. The sheet is then rinsed in PBS containing about 0.05% Tween 20.

Antibodies present in the patient's blood which are bound to the immobilized bacterial antigen, are detected by first incubating the nitrocellulose sheet for four minutes at room temperature in affinity purified goat anti-human IgG conjugated with horseradish peroxidase. After a brief washing step, the sheet is incubated for five minutes in a substrate solution of hydrogen peroxide and 4-chloro-1-naphthol, the latter substance being a chromophoric redox indicator. The sheet is then rinsed with water and blotted or air dried. A visible color change in a particular spot then indicates that a sufficient quantity of enzyme conjugated to the anti-human IgG was bound to the particular spot.

Comparative Example 1

Sheets prepared as described in Preparatory Example 1 were used to assay blood from normal subjects and from subjects having known periodontal disease. The sensitivity and specificity of the assay is tabulated in Table 1 below.

TABLE 1

Bacteria	Sensitivity	Specificity	Predictive Positive Value	Predictive Negative Value
Aa	0.800	0.844	0.800	0.844
Pg	0.923	0.864	0.800	0.950
Pi	0.857	0.952	0.923	0.909
Ec	1.000	0.800	0.727	1.000
Wr	0.833	0.941	0.833	0.942

As shown in Table 1, the present method exhibited sensitivity and specificity ranging from 0.8 to 1.0 with predictive values of positive and negative results ranging from 0.727 to 1.0. This study was performed using only five bacteria from the proposed bacterial battery and without the preferred serotype, thereby resulting in a lower sensitivity and predictive value for negative test.

Example 1

Prepare a dot blot apparatus by placing two blotters under the nitrocellulose sheet. Notch the corner of the sheet to identify top and bottom.

Spot a 9 x 12 cm nitrocellulose sheet with 10 μl of 5 x 107 dilution of each of the six different strains (or groups of strains) of bacteria, for six spots each in six different lines and a known positive and negative control for six spots each in two different lines as illustrated below:

TABLE 2

	Positive Control	Pt. #1	Pt. #2	Pt. #3	Pt. #4	Pt. #5	Pt. #6	Negative Control
Aa,B	....	....	....	....	....	....	....	....
Pg,A	....	....	....	....	....	....	....	....
Pi	....	....	....	....	....	....	....	....
Ec	....	....	....	....	....	....	....	....
Cr	....	....	....	....	....	....	....	....

Incubate at room temperature for 15. minutes.

Wash sheet five tiles with phosphate buffered saline (.01M) with 0.05% Tween-20 (PBST) and allow to dry for 30 minutes. (When washing, fill the apparatus with solution, then loosen the screws before using the vacuum. Tighten the screws again after completing all five washes.)

Treat all spots with 10μl of patient sera diluted 1:200.

Incubate at room temperature for ten minutes.

Wash five times with PBST.

Dilute goat anti-human IgG and (H and L chains) Horseradish Peroxidase Conjugate 1:500 in phosphate buffered saline (PBS) (.01 M) with 1% BSA. (Use 10μl conjugate and incubate ten minutes at room temperature).

Wash five times with PBST. Tighten screws while the vacuum is running.

Remove sheet for dot-blotter and lay in glass container.

While applying vacuuming step 10, prepare substrate solutions #1 and #2 in separate containers. Pour together over the nitrocellulose sheet. Incubate ten minutes at room temperature to develop color.

Solution #1: 60 mg 4 - chloro - 1 - naphthol and
20 ml methanol
Swirl well to dissolve

Solution #2: 100 ml Tris buffered saline and .6 ml H_2O_2 Float sheet in deionized H_2O in separate bowl to stop reactions. Blot dry with blotter on bottom and using kimwipes.

Read and record results as +/- for each organism.

Positive (+) = positive will display intense staining over the slot.

Negative (-) = negatives will display minimal or blotchy staining over the slot.

The test is accepted if all positive controls are positive and all negative controls are negative. Rerun if controls do not agree.

All positive reactions are considered indicative of current or recent infection. Normals would be negative.

Example 2

A. Preparation of the Nitrocellulose

Cut 0.45μM nitrocellulose (S & S BA 85) into 9 x 13cm sheets.

Hydrate a cut sheet of nitrocellulose and a sheet of filter paper (Bio-Rad Bio-Dot) for 2 minutes in 100 ml of PBS.

A gasket support plate (Bio-Rad Bio-Dot) is then centered over the nitrocellulose.

Make light pencil marks on the left and top side of the nitrocellulose to mark the rows, 4 lanes of individual wells in the gasket support plate.

Beginning with the second row, third lane well of the gasket support plate, dispense 8-10μl aliguots of each whole cell antigen preparation to the wells of each row as follows:

Aa FDC Y4 5 x 10^7cells/well

Pg 33277 5 x 10^7cells/well

Pi 25611 5 x 10^7cells/well

Cr 33238 1 x 10^8cells/well

Ec 23834 2 x 10^7cells/well

Following a 10 minute incubation with antigen, remove the gasket support plate and wash the nitrocellulose in 100 ml PBS for 1 minute.

Remove the nitrocellulose from the PBS wash, place on a paper towel, and allow the air-dry for 10 minutes - covered.

Block the nitrocellulose for 100ml 5% milk/PBS (Sanalac) for 15 minutes.

Take the nitrocellulose sheet out of the blocking agent and wash in 100ml 0.05% Tween-20/PBS for 10 minutes.

Remove the nitrocellulose from the Tween/PBS wash, place on a paper towel and allow to air-dry overnight - covered.

Store the prepared sheets of nitrocellulose at room temperature and covered for at least 7 more days prior to assay.

B. Assay of Patient Samples

Rehydrate a prepared sheet of nitrocellulose and a sheet of filter paper (Bio-Rad Bio-Dot) for 2 minutes in 100 ml 0.05% Tween-20/PBS for 2 minutes.

Place the hydrated filter paper on a 16 x 10cm glass plate and center the wet nitrocellulose sheet over the filter paper.

A gasket support plate (Bio-Rad Bio-Dot) is then centered over the nitrocellulose lining up the pencil marks with the rows and lanes of the individual wells.

Beginning with the third lane well, second row of the gasket support plate, dispense 5 µl aliguots of control sera or patient capillary blood to the wells of each lane as follows:

Positive Control Sera	Patient Capillary 1 2 3 4 5	Positive Control Sera
1/25 in 0.05% Tween/PBS	1/50 in 0.05 Tween/PBS	1/25 in 0.05% Tween/PBS

Following a 10 minute incubation with antibody, remove the gasket support plate and wash the nitrocellulose in 100ml 0.05% Tween/PBS for 1 minute.

Fix the nitrocellulose in 10ml methanol diluted in 100ml of tris buffered saline (.01M Tris) for 5 minutes.

Place the nitrocellulose sheet in a leveled 41/2" x 61/2" acrylic dish and saturate with 5ml of a 1/400 dilution

of goat anti-human IgG HRP (Calbiochem #29670) in 1% BSA/PBS.

Remove the nitrocellulose from the acrylic dish and wash for 5 minutes in 100ml 0.05% Tween/PBS.

During this last wash, prepare two developing solutions as follows:

a. 60mg 4-Chloro-1-Napthol/20ml methanol

b. 120μl 30% H_2O_2/100ml Tween/PBS

Combine the two developing solutions in a 9" x 9" glass pan, place the nitrocellulose into the solution and incubate for a maximum of 10 minutes.

Remove the nitrocellulose from the development solution, place on a paper towel and allow the sheet to air-dry covered before scoring.

Comparative Example 2

Nitrocellulose sheets were prepared as described in Example 2 for dot-blot immunoassay (DB) using spots containing either (1) **A. actinomycetemcomitans**, serotypes B (e.g. strain Y4; ATCC 43718) or (2) all three serotype of **A. actinomycetemcomitans**. The sheets were used to assay blood from normal subjects and from subjects having known periodontal disease. The results of the dot blot assay were compared to ELISA assays.

The ELISA results were determined as the "gold standard" for antibody measurements and positive/negative reactivity based upon Ebersole, J.L. et al., **Oral Micro.**

Immunol. 2:53-59 (1987). The results are tabulated in Table 3 below.

TABLE 3

	ELISA	DB AaY4	DB 3 serotypes Aa
Positive Reaction	9	9	12
Negative Reaction	16	16	13

The results showed a 92% agreement with the serotype B strain of **A. actinomycetemcomitans**, while inclusion of all 3 serotypes of **A. actinomycetemcomitans** showed only a 75% agreement.

Comparative Example 3

Another comparison test was performed similar to comparative Example 2, except that the nitrocellulose sheets were prepared using spots containing either (1) **P. gingivalis, serotype A** (e.g. ATCC 33277) or (2) all three serotypes of **P. gingivalis**. The reaction results compared with ELISA are tabulated in Table 4 below.

TABLE 4

	ELISA	DB Pg33277	DB 3 serotype Pg
Positive Reaction	12	11	14
Negative Reaction	13	14	11

The serotype A strain of **P. gingivalis** showed a 92% agreement while inclusion of all 3 serotypes of **P. gingivalis** showed only a 84% agreement with ELISA.

Thus, the use of **A. actinomycetemcomitans, serotype B** and **P. gingivalis, serotype A** in accord with the present invention provide increased accuracy in assessment of elevated antibody levels in patient sera.

Comparative Example 4

Nitrocellulose sheets were prepared as described in Example 2, using all five bacterial antigens. Patient samples were tested using fixation with methanol and without fixation comparing the results to ELISA. The results are tabulated below in Table 5.

TABLE 5

	ELISA	DB w/o methanol	DB w methanol
Positive Reaction	40	61	42
Negative Reaction	85	64	83

The results show that when using the fixation step with methanol a 97% agreement is achieved between the ELISA and DB and without the fixation step (i.e. w/o methanol) only 55% agreement is obtained.

The invention has been described in detail with reference to the preferred embodiments thereof. However, it will be appreciated that, upon consideration of the disclosure herein including the examples, those skilled in the art may make modifications and improvements within the spirit and scope of the claims. For example, a plurality of substrates each having a single spot containing an immobilized periodontapathegenic bacterium in a predetermined concentration may be used in place of a single substrate having a plurality of discrete spots.

We claim:

1. A method for diagnosing periodontal disease, the method comprising:

providing a substrate coated with a plurality of discrete spots, each spot containing one type of an immobilized periodontopathogenic bacterium in a predetermined concentration;

contacting the immobilized bacterium with a sample containing blood from a patient suspected of having periodontal disease, thereby forming conjugates of antibodies with the immobilized bacterium; and

detecting the antibody conjugates;

wherein the spots containing immobilized bacterium comprise at least one spot containing **Actinobacillus actinomycetemcomitans, serotype B** or **Porphyromonas gingivalis, serotype A.**

2. The method of claim 1, further comprising, after the contacting step, fixing the antibodies from the sample to the bacterium antigens.

3. The method of claim 1, wherein the detecting step comprises contacting the antibody/bacterium conjugates with labelled anti-human sera, washing away unbound labelled anti-human sera and detecting the bound labelled anti-human sera.

4. The method of claim 3, wherein the detecting step comprises detecting a visible change.

5. The method of claim 1, wherein the detecting step comprises contacting the antibody/bacterium conjugates with labelled anti-human IgG.

6. The method of claim 5, wherein the anti-human IgG is labelled with an enzyme which is detected using a chromophore substrate that undergoes a visible change.

7. The method of claim 1, further comprising comparing the results of the detecting step with the results using a reference sample having a predetermined quantity of antibodies to specific bacterium.

8. The method of claim 1, wherein the spots containing immobilized bacterium comprise spots containing each of the following bacterium:

Actinobacillus actinomycetemcomitans, serotype B, Porphyromonas gingivalis, serotype A, Prevotella intermedia, Eikenella corrodens and Campylobacter rectus, one type of bacterium per spot.

9. The method of claim 8, further comprising, after the contact step, fixing the substrate with methanol, and wherein the detecting step comprises contacting the antibody/bacterium conjugates with an enzyme labelled anti-human IgG, and determining the presence of bound anti-human IgG using a chromophore substrate that undergoes a visible change in the presence of an enzyme.

10. A solid substrate for a dot-blot immunoassay comprising a predetermined arrangement of discrete spots,

each spot containing one type of an immobilized periodontopathogenic bacterium in a predetermined concentration;

wherein the spots containing immobilized bacterium comprise at least one spot containing **Actinobacillus actinomycetemcomitans, serotype B** or **Porphyromonas gingivalis, serotype A.**

11. The substrate of claim 10, wherein the spots containing immobilized bacterium comprise spots containing one of each of the following bacterium:

Actinobacillus actinomycetemcomitans, serotype B, Porphyromonas gingivalis, serotype A, Prevotella intermedia, Eikenella corrodens and Campylobacter rectus, one type of bacterium per spot.

12. A kit comprising the substrate of claim 10, a reference sample containing a predetermined quantity of antibodies to specific periodontopathogenic bacterium, and labelled anti-human IgG.

13. The kit of claim 12, wherein the anti-human IgG is labelled with an enzyme and the kit further contains a chromophore substrate that undergoes a visible change in the presence of the enzyme.

14. The kit of claim 13, wherein the enzyme is horseradish peroxidase and the kit contains a solution comprising (1) 4-chloro-1-naphtol and (2) hydrogen peroxide.

15. The kit of claim 12, further comprising methanol.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/11658

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/53, 33/569; C12Q 1/04

US CL :435/7.2, 7.9, 34, 39, 174, 177, 179, 805, 810, 970; 436/518, 530

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.2, 7.9, 34, 39, 174, 177, 179, 805, 810, 970; 436/518, 530

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Oral Microbiol Immunol, Volume 2, issued 1987, J.L. Ebersole et al., "Human Serum Antibody Responses to Oral Microorganisms, IV. Correlation with Homologous Infection," pages 53-59, especially the right-hand column of page 54 and the left-hand and center columns of page 55.	1-15
Y	J Periodontal Res, Volume 26 (3 Pt 1), issued May 1991, A. Nagata et al., "Serological Studies of Porphyromonas (Bacteroides) gingivalis and Correlation with enzyme activity," pages 184-190, abstract only: especially the first 5 lines.	1-15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 08 JANUARY 1994	Date of mailing of the international search report 04 FEB 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer TONI R. SCHEINER Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Anal Biochem, Volume 166, No. 1, issued October 1987, J.B. Sheffield et al., "A Solid-Phase Method for the Quantitation of Protein in the Presence of Sodium Dodecyl Sulfate and other Interfering Substances," pages 49-54, abstract only: especially lines 6-7.	2, 9-15
Y	Laboratory Techniques in Biochemistry and Molecular Biology: Practice and Theory of Enzyme Immunoassays, Volume 15, issued 1985, P. Tijssen, pages 314-315, especially lines 1-4 of page 315.	1-15

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG: Biosis, CAB Abstracts, Embase, Medline, Derwent
search terms: periodont?, gingiv?, microb?, campylobacter rectus, wolinella recta, bacteroides intermedius, prevotella intermedia, porphrymonas gingivalis, bacteroides gingivalis, methanol, antibod?